

A MISSION shRNA Screen to Identify Genes that Modulate Cellular Response to Paclitaxel



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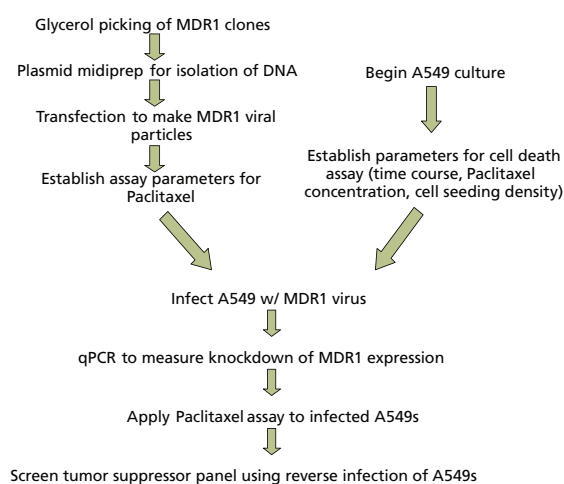
Abstract

An shRNA tumor suppressor panel was screened using reverse infection of an A549 tumorigenic cell line and then exposing it to a predetermined concentration of Paclitaxel, an anticancer drug. Genes identified led to an increased or decreased cell death after treatment with Paclitaxel when downregulated. In many cases, there were multiple constructs to a single gene that yielded positive hits. SMAD4, LZTS2, ST14, and VHL all increased the cell's sensitivity to Paclitaxel, while GLTSCR2, LATS1, NF1, PTEN, TP53, and WT1 had a protective effect. In addition to their potential significance as prognostic markers, these genes may represent significant pathways in tumorigenesis that have yet to be fully investigated.

Introduction

RNA interference (RNAi) is the mechanism of introducing a double-stranded RNA into a cell to target a specific gene of interest. An RNA-induced silencing complex will then unwind the strands of dsRNA and the associated antisense RNA strand bound to the complex will target homologous mRNA for cleavage and degradation, ultimately having a silencing effect in the targeted cell. In this experiment, short hairpin RNA (shRNA) in lentiviral vectors was used to generate gene knockdown. shRNA produces highly stable, long-term gene silencing and can integrate within even non-dividing cells. A human lung cancer cell line, A549, was transduced with 337 viruses targeting 74 genes. All of these genes are identified as tumor suppressors. Transduced cells were treated with Paclitaxel, and genes that induced a synergistic or protective effect on cell death were identified.

Overview



Results

MDR1 (human multi-drug resistant gene encoding for P-glycoprotein) shRNA lentivirus were produced and utilized to infect A549 cells. Each MDR1 construct: MDR1-1 (TRCN0000059683), MDR1-2 (TRCN0000059684), MDR1-3 (TRCN0000059685), MDR1-4 (TRCN0000059686), and MDR1-5 (TRCN0000059687) was measured by means of QRT-PCR and shown to be effective in knocking down transcription of the MDR1 gene. We believe that MDR1 knockdown will cause cells to become more sensitive to Paclitaxel. The results of the cell death assays confirm the idea and MDR1-2, the construct which demonstrated the greatest knockdown, was then used as a positive control for sensitizing cells to Paclitaxel. With all other cell assay conditions determined, a gene screen from a tumor suppressor panel was performed.

MDR1 Gene Percent Expression

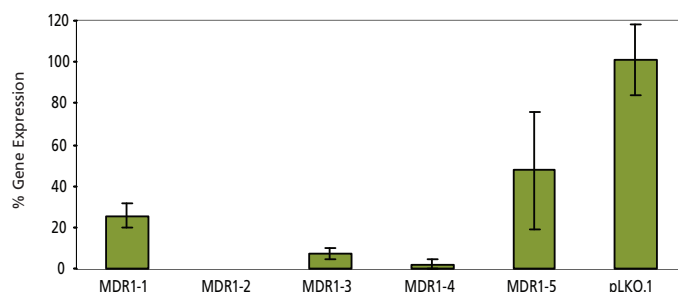


Figure 1: Quantitative RT-PCR was used to determine the efficacy of each shRNA construct to MDR1. Threshold values for construct MDR1-2 were below the level of detection. All values were normalized to the empty vector pLKO.1 control transduced cells.

Cell Death from Increasing Concentrations of Paclitaxel on MDR1 shRNA Infected A549

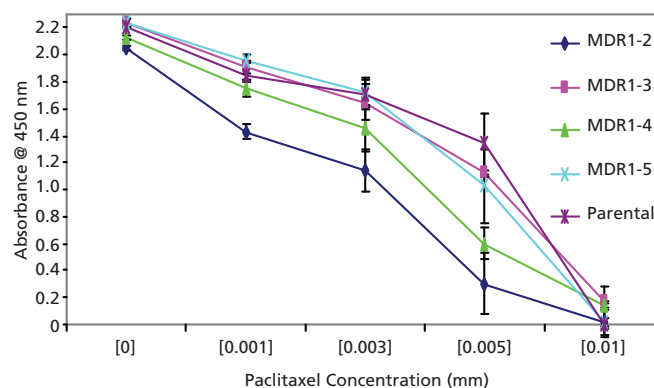


Figure 2: A549 cells were seeded at 40,000 cells/cm² in a 96-well plate and treated with increasing concentrations of Paclitaxel for 24 hours. The wells were then assayed with WST1 and read at 450 nm using SoftMax Pro software.

The screen yielded positives for increased resistance, as well as hits for increased sensitivity to Paclitaxel. TP53, GLTSCR2, LATS1, NF1, PTEN and WT1 were among some of the genes that indicated any transcript silencing would increase cellular resistance to drugs. This data reconfirms the literature findings on the main function of tumor suppressors. However, the genes that sensitized cells to the Paclitaxel were more novel and may open doorways for further investigation. These genes, LZTS2, SMAD4, ST14, and VHL all had constructs that were consistently showing up in the top 25 hits. Five out of five SMAD4 constructs fall into this category, which strongly demonstrates that it may be important in therapeutic efforts of treatment.

Comparison of Cell Survival from Paclitaxel vs. No Drug Treatment

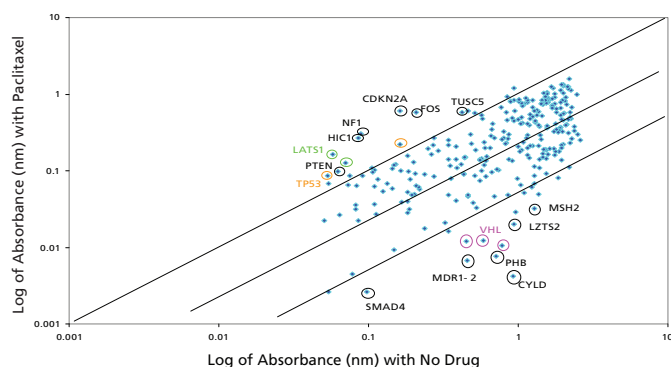


Figure 3: Cell death was due to a 5 mM concentration of Paclitaxel over a period of 24 hours. Outliers from the screen are genes that either make cells more sensitive or resistant to Paclitaxel. Genes noted here effectively correspond to the ratios determined in figures 4 and 5.

Gene Knockdowns Conferring in Enhanced Sensitivity to Paclitaxel

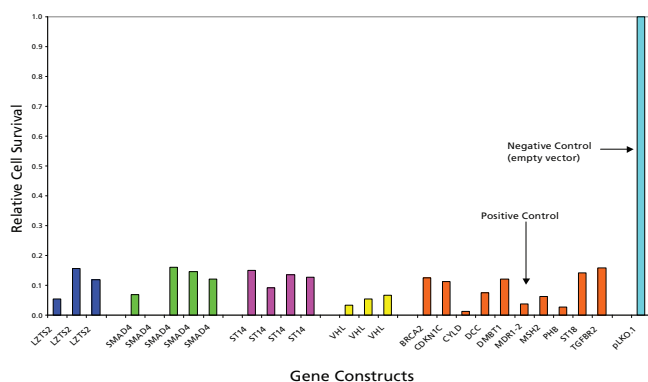


Figure 4: A ratio was established between cell survival in drug versus no drug treatment and the top 25 most sensitizing constructs were recorded, as compared to the negative control, pLKO.1, normalized to 1. Knocking down these genes causes cells to be more sensitive to Paclitaxel.

Gene Knockdowns Conferring in Most Resistance to Paclitaxel

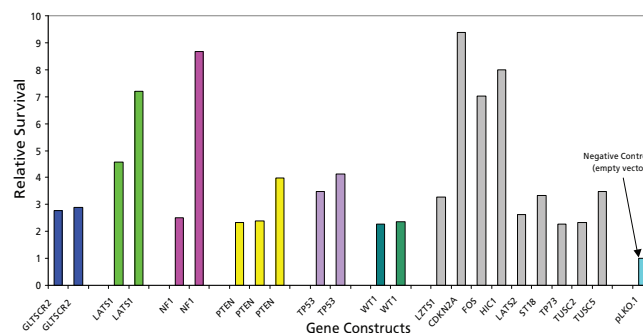


Figure 5: A ratio was established between cell survival in drug versus no drug treatment and the top 25 least sensitizing constructs were recorded, as compared to the negative control, pLKO.1 normalized to 1. Knocking down these genes causes cells to be more resistant to Paclitaxel.

Materials and Methods

Cell Culture

The human lung adenocarcinoma cell line A549 was obtained from ATCC (Manassas, VA). The cell line was cultured in F12 Ham's media supplemented with 10% v/v fetal bovine serum, 4mM final L-glutamine, penicillin and streptomycin (all obtained from Sigma-Aldrich) in T-75 cm² cell culture flasks, at 37 °C and 5% CO₂. At ~80% confluency, the cells are trypsinized and reseeded into 96-well plates for assay.

Cytotoxicity Assays

In vitro cytotoxicity was performed using a Quick Cell Proliferation Assay Kit (BioVision, Mountain View, CA). Cells were plated overnight in 96-well plates at 40,000 cells/cm². Then increasing concentrations of Paclitaxel in F12 Ham's media replaced the normal media. After 24 hours of culture with the chemotherapeutic drug, 10 ml of WST-1 was added to each well and the plates were incubated an additional 4 hours. The formazan dye produced by live cells can then be read by a spectrophotometer for absorbance at 450 nm. Absorbance measurements were normalized by subtracting the value of blank wells from the treated wells.

Plasmid Midiprep

Isolation of the plasmid DNA from MDR1 constructs was performed using GenElute HP Plasmid Midiprep kit (Sigma- Aldrich, St. Louis, MO). Refer to instruction manual for the appropriate protocol. DNA was normalized to 20 ml/ mL in a 1:10 dilution of DNA and TE, and analyzed using SoftMaxPro computer software.

Transfection

FuGENE6 Transfection Reagent was used, along with packaging construct (pDelta 8.9) and envelope construct (pCMV-VSV-G) in serum free DME media for all transfections. Virus particles were harvested at 40 and 48 hours post-transfection, yielding approximately 200 ml per sample. A p24 assay was performed to test for quality, using an HIV-1 p24 Antigen ELISA manual kit (Gentaur, Brussels, Belgium).

Infection

A549 cells were infected at a MOI of 10. 40,000 cells/ well were seeded in a 24-well plate. Empty vector (pLKO.1) was a positive control for puromycin selection as well as negative control for MDR1 knockdown. Four replicates of each construct were infected and duplicates were made of the control and blank wells. Final concentration of polybrene used was at 8 mg/ mL. Cells were selected with puromycin at 48 hours post-infection.

Reverse Infection

Reverse infection was performed on A549 with 5 ml of virus from a tumor suppressor panel at a seeding density of 16,000 cells/ well in a 96-well plate. Triplicates of pLKO.1 virus was included as a negative control, and MDR1-2 virus as a positive control. Final concentration of polybrene at 8 mg/ mL was added and 3 mg/ mL of puromycin was used to select at 48 hours post-infection.

Quantitative RT- PCR

RNA from the infected A549 cell lines was harvested using GenElute Mammalian Total RNA Kit (Sigma-Aldrich, St. Louis, MO). TaqMan Gene Expression Assays primer and probe, ABCB1, were used (Applied Biosystems, Foster City, CA). MDR1 primers were 5'- labeled with FAM reporter dye and 3'-labeled with fluorescent quencher. QRT-PCR was performed using a Master Mix kit prepared with Sigma's quantitative RT-PCR ReadyMix (QRO200) supplemented with MgCl₂ and water. Reference dye was also included as an internal control for fluorescence. 20 ml total reactions were set up, using 4 ml of RNA. All reactions were run and analyzed with the Mx3000 qPCR system and software (Stratagene, La Jolla, CA). Reaction conditions were set up at: 15 min at 42 °C, 3 min at 94 °C, and 45 cycles of 15 s at 94 °C and 1 min at 60 °C. mRNA expression from the MDR1 constructs were compared against GAPDH mRNA for quantification. Assays were performed in triplicate (including the empty vector construct), as well as two no-template controls, and one well each of no reverse transcriptase mix. Values are expressed with pLKO.1 expression normalized to 100%.

Conclusion

shRNA is a powerful tool for long term gene silencing, making it useful for various types of screens. This screen has effectively identified genes that may be prognostic for chemotherapeutic responses, such as GLTSCR2, LATS1, NF1, PTEN, and TP53. It may have also identified potential targets for enhancing the drug effects of Paclitaxel, including SMAD4, LZTS2, ST14, and VHL. This is of interest in the area of cancer biology, where the development of more focused treatments rather than the standard targeting of all dividing cells could be beneficial. Since Paclitaxel has such a varied effect depending on the genetic composition of the tumor, later work may be performed using a different cell line or another drug similar to its effects in order to identify additional genes involved in modulating drug sensitivity.